Applied and Environmental Microbiology, Dec. 2009, p. 7409–7416 Vol. 75, No. 23
0099-2240/09/$12.00 doi:10.1128/AEM.01400-09

Influence of Different Filling, Cooling, and Storage Conditions on the Growth of *Alicyclobacillus acidoterrestris* CRA7152 in Orange Juice▽

Ana Cláudia N. F. Spinelli,[1] Anderson S. Sant'Ana,[1,2] Salatir Rodrigues-Junior,[3] and Pilar R. Massaguer[1,4]*

Department of Food Science, Faculty of Food Engineering, State University of Campinas, Campinas, SP, Brazil[1]; Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil[2]; Department of Rural Engineering, Federal University of Espírito Santo, Alegre, ES, Brazil[3]; and Department of Chemical Process, Faculty of Chemical Engineering, State University of Campinas, Campinas, SP, Brazil[4]

Received 15 June 2009/Accepted 25 September 2009

The prevention of spoilage by *Alicyclobacillus acidoterrestris* is a current challenge for fruit juice and beverage industries worldwide due to the bacterium's acidothermophilic growth capability, heat resistance, and spoilage potential. This study examined the effect of storage temperature on *A. acidoterrestris* growth in hot-filled orange juice. The evolution of the *A. acidoterrestris* population was monitored under six different storage conditions after pasteurization (at 92°C for 10 s), maintenance at 85°C for 150 s, and cooling with water spray to 35°C in about 30 min and using two inoculum levels: $<10^1$ and 10^1 spores/ml. Final cooling and storage conditions were as follows: treatment 1, 30°C for the bottle cold point and storage at 35°C; treatment 2, 30°C for 48 h and storage at 35°C; treatment 3, 25°C for the bottle cold point and storage at 35°C; treatment 4, 25°C for 48 h and storage at 35°C; treatment 5, storage at 20°C (control); and treatment 6, filling and storage at 25°C. It was found that only in treatment 5 did the population remain inhibited during the 6 months of orange juice shelf life. By examining treatments 1 to 4, it was observed that *A. acidoterrestris* predicted growth parameters were significantly influenced ($P < 0.05$) either by inoculum level or cooling and storage conditions. The time required to reach a 10^4 CFU/ml population of *A. acidoterrestris* was considered to be an adequate parameter to indicate orange juice spoilage by *A. acidoterrestris*. Therefore, hot-filled orange juice should be stored at or below 20°C to avoid spoilage by this microorganism. This procedure can be considered a safe and inexpensive alternative to other treatments proposed earlier.

The first *Alicyclobacillus* sp. discovered was isolated in 1982 from spoiled apple juice aseptically packed in Germany and was considered at that time strictly limited to thermophilic and acidic environments (5). The spoilage of fruit juices by *Alicyclobacillus* is characterized by "off" flavors (medicinal or phenolic) due to guaiacol, 2,6-dibromophenol, and 2,6-dichlorophenol (20, 32, 25). As the spoilage does not show any evident signs like swelling of the container or any overt changes in the fruit juice (e.g., pH or turbidity), it is often not recognized until the packages are opened, the product is tasted, and consumer complaints are received by the manufacturer (6).

Alicyclobacillus acidoterrestris, *Alicyclobacillus cycloheptanicus* and *Alicyclobacillus acidocaldarius* were the first three species described when the *Alicyclobacillus* genus was created in 1992 (31). Although there are currently more than 15 species described (27), only four *Alicyclobacillus* species (*A. acidoterrestris*, *A. pomorum*, *A. herbarius* and *A. acidophilus*) have shown the ability to produce off flavors in fruit juices or beverages (5, 13, 9, 1). Of these, *A. acidoterrestris* is considered the most important spoilage species within the *Alicyclobacillus* genus either by its frequency of occurrence or by its linkage to the spoilage problems of fruit juices and beverages. The broad temperature range for *A. acidoterrestris* growth (25 to 60°C) (33, 21, 20, 30, 11), its ability to grow under acidic environments (pH 2.5 to 6.0) (20, 30, 19, 22, 6), and its high heat resistance in orange juice (*D* at 95°C of 2.7 min) (8) together provide adequate conditions for both survival through pasteurization and growth during juice storage.

A. acidoterrestris growth and the consequent orange juice spoilage can lead to enormous economic losses; therefore, this microorganism is currently considered a major challenge for the fruit juice industries. It is known to be difficult, if not impossible, to guarantee the absence of *Alicyclobacillus* spores on the surface of fruits used to make juices since the soil is the primary niche of *Alicyclobacillus* spp. (7). Subsequently, control measures such as avoiding fruit contact with soil and the use of sanitizers during the fruit washing step before crushing have been studied (16, 12). In addition, fruit juice producers need to better control the pasteurization conditions and to redesign their thermal processes for targeting *Alicyclobacillus* spp. (28, 24). However, the limited effectiveness of sanitizers against *Alicyclobacillus* spores and the sensory and nutritional problems that may arise from increased time and temperature regimes in pasteurization are recognized. Since the complete inactivation of *Alicyclobacillus* spores from raw materials may not be feasible and since juice spoilage by this microorganism depends on the germination of spores and outgrowth, studies reporting conditions that avoid spoilage by controlling *Alicyclobacillus* spore germination are necessary. The best option to manage the challenge that *Alicyclobacillus* presents to the fruit juice industries will be one that results in the fewest alterations in processing and storage conditions, preserves the nutritional

* Corresponding author. Mailing address: LABTERMO—Fundação André Tosello, Rua Latino Coelho 1301, Parque Taquaral, CEP 13087-010 Campinas, SP, Brazil. Phone and fax: 55 19 3213 1501. E-mail: pilar.rodriguez@terra.com.br.

▽ Published ahead of print on 2 October 2009.

and sensory aspects of the final products, does not impact production costs and commercial practices, and at the same time ensures the control of the microorganism.

In the fruit juice industry, two main types of thermal processes are commonly applied: pasteurization followed by a hot-fill process or pasteurization followed by a cold-fill process (6). In the former, after the product is heated to >90 to 95°C, it is held hot for 15 to 20 s. As the temperature decreases to 82 to 84°C, the product is filled into the package. Next, the product is held for approximately 2 min before the packages are cooled to room temperature. Hot filling has been extensively used in the manufacturing processes of fruit-based drinks and beverages, but problems due to spoilage caused by *Alicyclobacillus* may arise during fruit juice shelf life. This is due to the extended time that the product is maintained at temperature conditions adequate for the germination and outgrowth of acidothermophilic spore-forming microbes. Despite several studies regarding the factors that affect *A. acidoterrestris* growth and heat resistance (12, 18, 2, 29), there is a lack of research on the effects of hot-filled fruit juice storage conditions on *A. acidoterrestris* growth during juice storage. Thus, this study aimed at estimating and comparing the growth parameters (maximum population ratio, κ; lag time, λ; and maximum growth rate, μ) of *A. acidoterrestris* survival in hot-filled orange juice that was cooled and stored under several conditions that simulate industrial and commercial practices. Primary growth parameters were estimated by using the Baranyi predictive model (3). Additionally, orange juice cooling and storage conditions that avoided germination, growth, and guaiacol production by *A. acidoterrestris* CRA 7152 were determined.

MATERIALS AND METHODS

Orange juice. Frozen concentrated orange juice (pH 3.8 and 66.17°Brix) supplied by a Brazilian orange juice producer was reconstituted to 11°Brix, pH 3.5, with sterile distilled water.

Microorganism and spore suspension preparation. The *A. acidoterrestris* CRA 7152 strain (recognized as a guaiacol producer) was provided by Danisco Cultor. For preparation of the *A. acidoterrestris* spore suspension, vegetative cells were initially grown in six plates containing potato dextrose agar, pH 4.0 (Oxoid), and incubated at 45°C for 5 days. Grown cells were then collected from the plates with sterile glass rods with 6 ml of sterile distilled water per plate and transferred to three sterile screw-cap tubes (16 by 250 mm). Spores were activated at 80°C for 10 min, followed by fast cooling in an ice bath until room temperature was reached (12). An aliquot (0.2 ml) of activated suspension was inoculated into 100 glass bottles (290 ml), each containing 70.0 ml of solidified and slanted *A. acidocaldarius* medium (33). These bottles were incubated for 7 days at 45°C. Spores were collected after 90% sporulation, which was confirmed microscopically (14). The collected spores were washed and resuspended in sterile distilled water after each of four centrifugations, two at 1,500 × *g* for 10 min and two at 6,160 × *g*, all at 4°C for 10 min. Lysozyme at 0.15 mg/ml was added after the second washing, and the pH was adjusted to 11 for the disruption of vegetative cells (26). Spore suspension was stored at 4°C until use. Viable spores were enumerated by pour-plating on yeast extract soluble starch (YSG) agar (g/liter: yeast extract, 2; soluble starch, 2; glucose, 1; agar, 1.5) and *Alicyclobacillus* medium (Merck, Darmstadt) after thermal activations for 20 min at 70°C and for 10 min at 80°C, respectively (9, 10). The inverted plates were incubated at 45°C for 5 days. Concentration of the spore suspension was 9.7×10^8 spores/ml for *Bacillus acidoterrestris* thermophilic agar and 9.4×10^8 spores/ml for YSG agar.

Hot-filling processing. A Microthermics ultrahigh temperature-high temperature/short time unit (LAB-DH-25; Microthermics, NC) equipped with a spiral indirect-type tubular heat exchanger was used. After the juice was heated to 92°C and held at this temperature for 10 s, it was filled at 85°C through a sterile product outlet (SPO) in a class 100 laminar flow (Microthermics, NC). The unit was supplied with thermocouples (Omega T-type needle) and a data logger for thermal data acquisition (Fluke Hydra model 2625A) over the entire processing period. The flow rate applied was 1.7 liters/min. Thermocouples were located at the end of the preheating, holding, cooling, SPO, and cooling (by water spraying) units, and the temperature was recorded every 10 s. In addition, two pressure sensors were installed, the first at the preheater and the second at the cooler. A schematic figure of the Microthermics equipment used can be seen in Sant'Ana et al. (22).

Industrial conditions were simulated by processing the juice at 92°C for 10 s, followed by filling at 85°C, bottle inversion for 20 s (for bottle cap disinfection), maintenance at 85°C for 150 s, and cooling to 35°C in about 30 min by water spraying. The juice bottles were kept at 85°C for 150 s in a temperature-controlled water bath in order to simulate surge tank maintenance before filling. Immediately before high temperature/short time-system feeding, the juice was inoculated with $<10^1$ or 10^1 *A. acidoterrestris* nonactivated spores per ml. After each process, a clean-in-place procedure was conducted as suggested by Ecolab.

Polyethylene terephthalate bottles (500 ml) with an outside diameter of 60 mm and a height of 205.6 mm and equipped with screw caps were used. The bottles and the caps were disinfected with 0.3% and 0.05% solutions (vol/vol) of peracetic acid (P3 Oxônia from ECOLAB, Brazil) in sterile water, respectively. After the contact time and the discharge of the peracetic solution, both bottles and caps were washed two times with sterile distilled water and then locked in sterile bags for further use (19). This procedure took place 1 day before juice processing. The capping process was carried out in an aseptic filling unit (SPO) (class 100 laminar flow chamber). The caps were placed on the bottlenecks with the aid of a sterile pincer and closed manually.

Two sensors (PT 1000 and TMI-Orion NanoVACQ-L/1Tc) were inserted at the bottle cold point in two different bottles. Each sensor was installed in a bottle at the beginning and at the end of the filling process for each orange juice lot. This permitted temperature monitoring of filling, cooling, and shelf life temperature. The readings were collected at intervals of 10 s over the 3 h after filling. After that, they were collected at intervals of 1 h until the end of the storage cycle. Three flexible-type T thermocouples (Copper-Constantan) (TT-T36-Omega) were placed in the bottle before filling to collect data during water spray cooling.

Storage conditions and monitoring of *A. acidoterrestris* population in orange juice. The evolution of the *A. acidoterrestris* population was monitored under six different filling/storage conditions. The treatments were as follows: treatment 1, cooling down to 30°C at the bottle cold point followed by storage at 35°C; treatment 2, cooling down to 30°C for 48 h followed by storage at 35°C; treatment 3, cooling down to 25°C at the bottle cold point followed by storage at 35°C; treatment 4, cooling down to 25°C for 48 h followed by storage at 35°C; treatment 5, cooling down to 20°C during shelf life (6 months); and treatment 6, filling and storage at 25°C. For each condition two different levels of inoculum were applied: $<10^1$ and 10^1 spores/ml of orange juice; juice without inoculum was used as the control. Treatment 6 was examined with only a single inoculum of 10^1 spores/ml in order to evaluate if both filling and storage at 25°C could inhibit growth of *A. acidoterrestris*. Conditions 1 and 3 correspond to hot filling with quick cooling, while conditions 2 and 4 correspond to hot filling with slow cooling. In summary, in the treatments 1 to 5 the juice was hot filled while in treatment 6 it was cold filled.

After each experiment, at least 10 juice bottles were aseptically opened, subjected to serial 10-fold dilutions, and pour-plated into YSG medium, followed by incubation at 45°C for 5 days. Orange juice bottles from each treatment were taken and used to follow the time course of *A. acidoterrestris* growth by periodically sampling and counting in YSG medium. Growth curves were monitored until the microorganism reached the stationary phase under the different conditions studied. A period of 288 h (12 days) was established as a maximum time for analyzing control samples (without inoculum) since the juice was free from *A. acidoterrestris* spores, according to a previous microbiological examination. This was the maximum time for *A. acidoterrestris* to reach stationary phase and allowed monitoring of the growth parameters under most of the storage conditions (1 to 4) with both inoculum levels ($<10^1$ and 10^1 CFU/ml). On the other hand, treatments 5 and 6 were monitored over 6 months, the expected hot-filled orange juice shelf life. The time intervals for monitoring *A. acidoterrestris* growth were established based on previous experiments, which indicated a generation time of approximately 8 h.

Determination of growth parameters. Growth data were analyzed by the DMFit program (www.ifr.ac.uk/safety/DMfit) (3). The following bacterial growth kinetic parameters were estimated: lag time, λ (days); maximum growth rate, μ; and maximum population ratio, κ, which represents the ratio of maximum concentration ($\log N$) to the inoculum ($\log N_0$), where N is the population at time t and N_0 is the initial population. By analyzing every growth curve, it was estimated that a 10^4 CFU/ml population of *A. acidoterrestris* was required to produce guaiacol detectable by a Kirin kit. Therefore, the time to reach this population

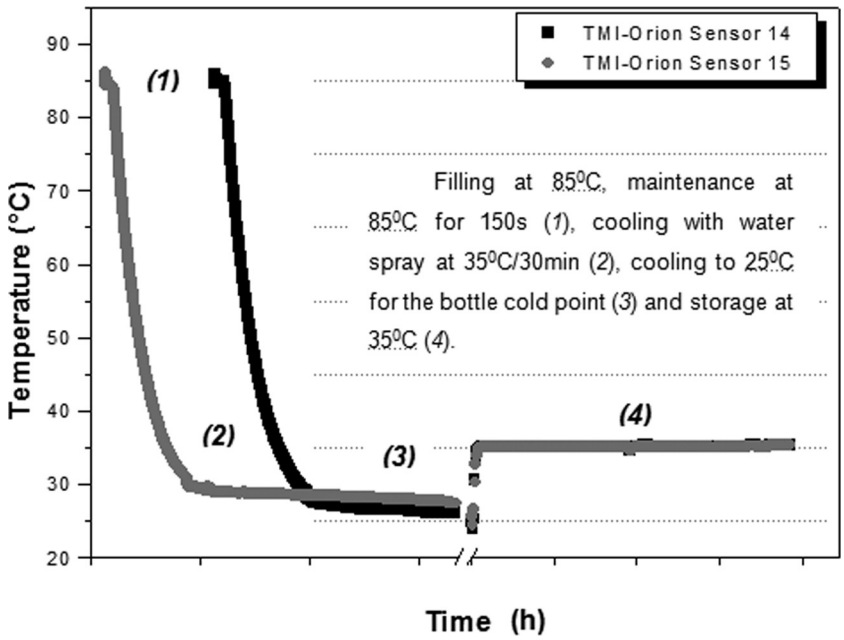

FIG. 1. Cooling thermal profile for treatment 3. Numbers 1 to 4 indicate the different stages in which bottles within this treatment were submitted. TMI Orion sensor 14 shows the temperature history profile from the beginning, and TMI Orion sensor 15 is from the ending of the process. Values on the time scale were not shown because sensors were used at different times within the process.

was established and identified as $t10^4$ for every storage condition. This value was considered critical for evidence of orange juice spoilage.

Guaiacol detection. The Kirin kit method for guaiacol detection developed by Niwa and Kuriyama (15) was used. As spoilage strains of *A. acidoterrestris* carry the *vdc* gene responsible for guaiacol production, the method is based on the reaction of vanillic acid (a guaiacol precursor) with a peroxidase enzyme. This results in the formation of a dark brown compound, allowing easy visual judgment. The method has a guaiacol detection sensitivity of 25 μg/ml. This test was applied to every sample as a measure of *A. acidoterrestris* growth.

Statistical analysis. Descriptive statistical calculations were applied to the data in order to determine the mean and standard deviation. Analysis of variance with a Tukey test was used at the 5% significance level to check for significant statistical differences among treatments, using Statistica, version 7.0 (Statsoft).

RESULTS

Typical thermal profiles after heating (hot-fill, cooling, and storage), corresponding to treatment 3 (25°C for the bottle cold point followed by storage at 35°C), were very similar for both bottles (Fig. 1). These thermal profiles were obtained at the start and at the end of all processes (1 to 6), showing rigorous temperature control during the experiments.

Table 1 shows the Baranyi estimates of growth parameters for every proposed treatment with the two inoculum levels studied. Data indicated that most of the *A. acidoterrestris* pre-

TABLE 1. Predicted growth parameters for *A. acidoterrestris* in hot-filled orange juice stored under various conditions[a]

Treatment no.[b]	Description	Treatment conditions	Inoculum level (spores/ml)	λ (h)	μ log [(CFU/ml)/h]	κ	$t10^4$ (h)
1	Hot filling with quick cooling	Cooling to 30°C at the bottle cold point and storage at 35°C	$<10^1$	51.71 ± 3.73 DE	0.093 ± 0.0085 ABC	4.20 ± 0.12 A	81 ± 1.4 EF
			10^1	62.73 ± 5.18 CD	0.104 ± 0.0332 AB	3.26 ± 0.01 BC	84 ± 5.7 DEF
2	Hot filling with slow cooling	Cooling to 30°C for 48 h and storage at 35°C	$<10^1$	75.19 ± 4.00 C	0.076 ± 0.01 BC	4.44 ± 0.05 A	116 ± 5.7 ABC
			10^1	74.25 ± 11.31 C	0.091 ± 0.0071 BC	3.52 ± 0.20 B	104 ± 5.7 BCD
3	Hot filling with quick cooling	Cooling to 25°C at the bottle cold point and storage at 35°C	$<10^1$	53.90 ± 3.51 DE	0.079 ± 0.0078 BC	3.56 ± 0.17 B	95 ± 1.4 CDE
			10^1	41.14 ± 3.98 E	0.084 ± 0.0014 BC	2.69 ± 0.08 D	67 ± 1.4 F
4	Hot filling with slow cooling	Cooling to 25°C for 48 h and storage at 35°C	$<10^1$	100.4 ± 0.40 B	0.101 ± 0.0078 ABC	3.57 ± 0.13 B	132 ± 0.0 A
			10^1	105.54 ± 1.22 B	0.149 ± 0.0226 A	2.90 ± 0.08 CD	125 ± 12.7 AB
6	Cold filling	Filling and storage at 25°C	10^1	270.95 ± 3.18 A	0.044 ± 0.0057 C	1.85 ± 0.01 E	—[c]

[a] Values are means ± standard deviations. Different capital letters in the same column indicate significant statistical differences according to a Tukey test ($P < 0.05$).
[b] Control samples for treatments 1 to 4 were stored for 288 h, and for treatments 5 and 6, they were stored for 6 months. Data on treatment 5 were not included since no growth was observed during the 6 months.
[c] Maximum population of *A. acidoterrestris* in orange juice did not reach 10^4 CFU/ml after 6 months of storage.

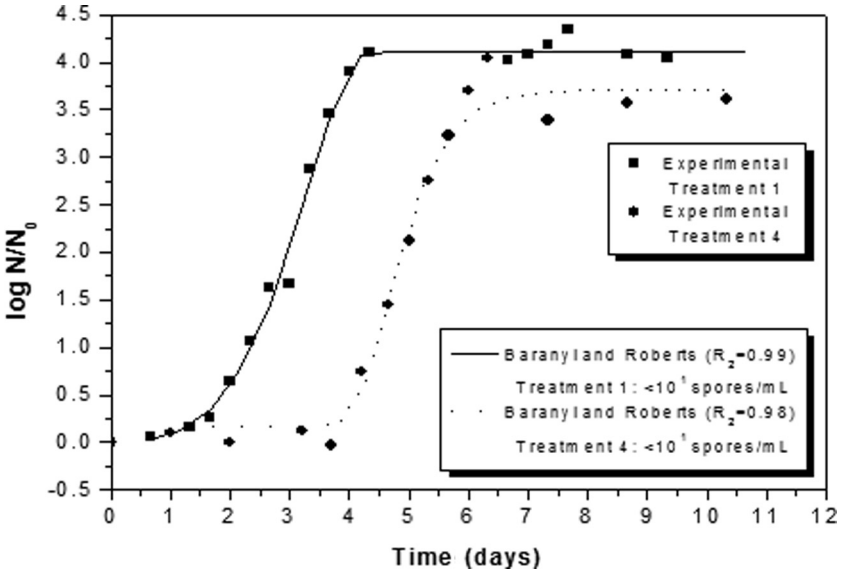

FIG. 2. Experimental data and fitted models obtained for treatment 1 (cooling down to 30°C at the bottle cold point followed by storage at 35°C) and treatment 4 (cooling down to 25°C for 48 h followed by storage at 35°C) at the same inoculum level ($<10^1$ spores/ml).

dicted growth parameters were significantly influenced ($P < 0.05$) by either inoculum level or cooling/storage conditions. Overall, hot filling with quick cooling yielded lower λ, $t10^4$, and κ values than hot filling with slow cooling. The value of μ was not significantly affected by the different treatments studied, except for treatment 1 with 10^1 spores/ml and treatment 6. Fig. 2 shows a comparison between treatments 1 (faster-cooling condition) and 4 (slower-cooling condition), both inoculated with $<10^1$ spores/ml. This figure illustrates the effect of slow-cooling conditions in increasing λ ($P > 0.05$) and κ values for *A. acidoterrestris*.

In Table 2 it can be seen that the time required to reach the target temperature during cooling was very close for both the quick and slow treatments. Despite this, the time to reach the target storage temperature (35°C) could be considered primarily responsible for the result that the λ and $t10^4$ values ($P < 0.05$) of treatment 3 were almost half those of treatment 4.

Lag time, λ, differed significantly ($P < 0.05$) among all the treatments but not between the two inoculum levels in the same treatment. Treatment 3 (at $<10^1$ spores/ml) did not show a significant difference ($P > 0.05$) compared with the two spore levels of treatment 1. It was observed that hot-filling with slow-cooling treatments (2 and 4) yielded higher λ values than hot-filling with quick-cooling treatments (1 and 3). A λ value of up to six times higher than the others was obtained when cold filling was applied ($P < 0.05$).

In contrast to the λ value, the *A. acidoterrestris* growth rate (μ) in cold-filled orange juice (treatment 6) was different ($P < 0.05$) only from treatments 1 and 4, which are, respectively, hot filling with quick cooling and slow cooling, with an inoculum level of 10^1 spores/ml. Again, no significant difference ($P > 0.05$) between spore inoculum levels in the same treatment was found. However, hot-filled orange juice cooling rates did not significantly affect ($P > 0.05$) the growth rate for treatments 1 and 4.

The data in Table 1 show that the higher the spore inoculum, the lower the κ values. This is because κ values are the difference between the log of the maximum concentration (N_f) and the inoculum (N_0). Therefore, κ is smaller for higher inoculum levels or when the maximum concentration is not reached (treatment 6, filling and storage at 25°C).

In treatment 5, hot-filled orange juice inoculated with $<10^1$ and 10^1 spores/ml of *A. acidoterrestris* showed no growth (Fig. 3). Thus, guaiacol was not detected during the product shelf life (6 months). In contrast, in treatment 6 the counts of this microorganism remained stable until 240 h, when growth was observed. After this time interval, the count reached 10^3 CFU/ml but did not exceed 10^4 CFU/ml (Fig. 4). Thus, $t10^4$ values could

TABLE 2. Time to reach the final temperature in orange juices processed by various treatments

Treatment no.[a]	Description	Cooling target temp and time point	Mean time to reach target temp during cooling (h)	Mean time to reach storage temp of 35°C (h)
1	Hot filling with quick cooling	30°C at the bottle cold point	0.66	17
2	Hot filling with slow cooling	30°C for 48 h	0.58	18
3	Hot filling with quick cooling	25°C at the bottle cold point	3.62	8
4	Hot filling with slow cooling	25°C for 48 h	2.95	32

[a] Data on treatment 5 were not included since no growth was observed during the 6-month storage period.

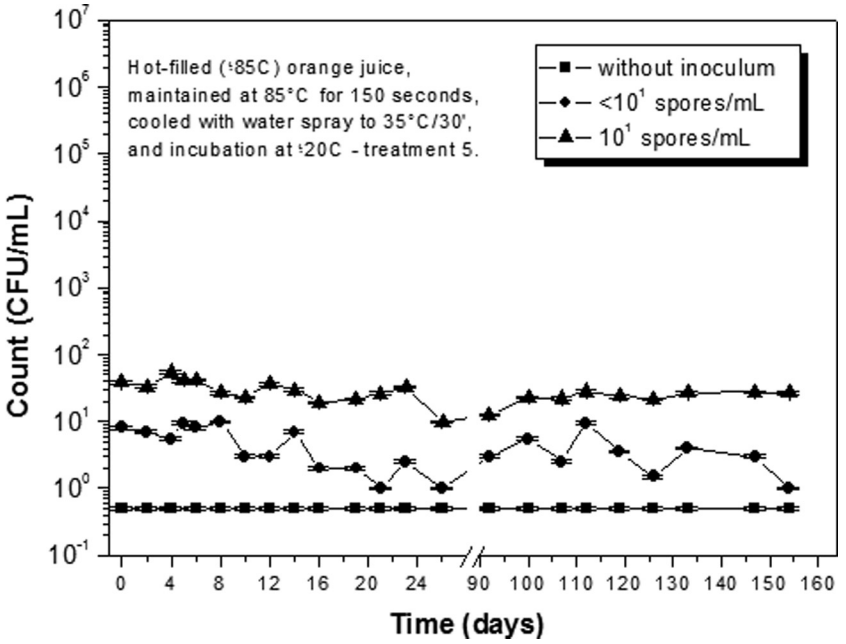

FIG. 3. *A. acidoterrestris* growth curve for treatment 5 with maintenance at 20°C during a shelf-life of 6 months.

not be determined for these two conditions. As expected, higher $t10^4$ values were obtained for hot-filling treatments with slow cooling, either for 25°C or 30°C, which were also associated with the higher λ values. On the other hand, treatment 1 yielded the lowest $t10^4$ values. The data indicated that for treatments 1, 2 and 4, the inoculum level did not significantly affect ($P > 0.05$) the $t10^4$ values obtained.

A comparison between two different inoculum levels in the same treatment, treatment 4 (cooling down to 25°C for 48 h followed by storage at 35°C), is shown in Fig. 5.

DISCUSSION

In this study, several combinations of cooling temperatures were applied in industrial orange juice hot-filling processes. The growth parameters of *A. acidoterrestris* were estimated by applying the Baranyi model to the experimental data to determine the best conditions for avoiding the growth of *A. acidoterrestris* and orange juice spoilage. This is the first report on predicted growth parameters of *A. acidoterrestris* as affected by orange juice filling and storage temperature. As elimination of

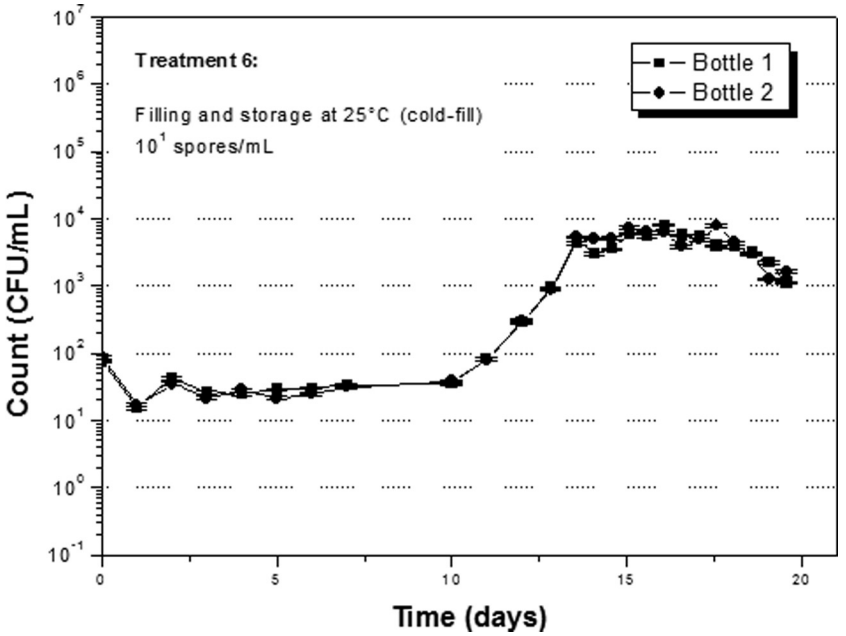

FIG. 4. *A. acidoterrestris* experimental growth curve for treatment 6.

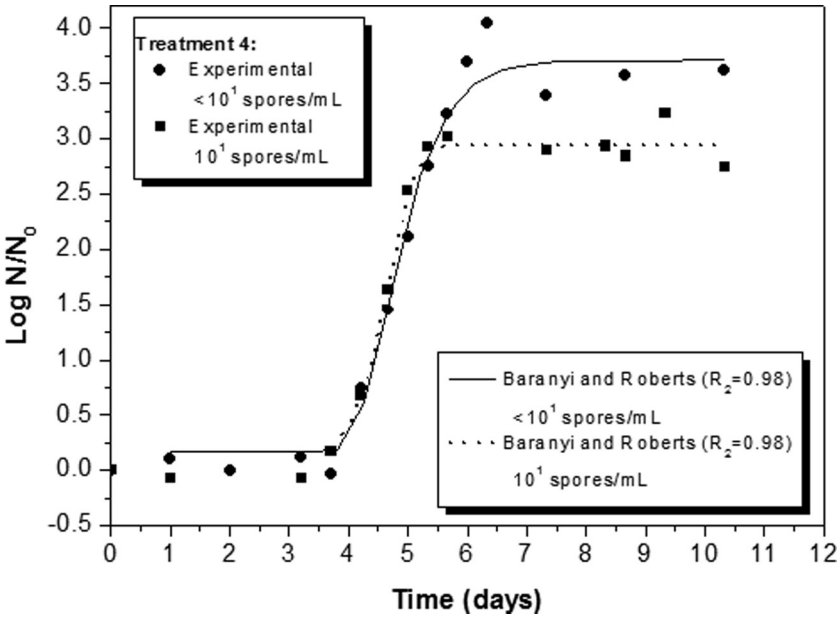

FIG. 5. Experimental data and fitted models obtained for treatment 4 (cooling down to 25°C for 48 h followed by storage at 35°C).

A. acidoterrestris from fruits destined for hot-fill juice processing and, consequently, elimination of risks for spoilage episodes are not achievable in industrial practice, here we report and experimentally demonstrate for the first time the complete and partial inhibition of this microorganism when orange juice is stored at temperatures of 20°C and 25°C, respectively.

As can be seen in Table 1, quick-cooling conditions would lead to a reduction of λ, κ, and $t10^4$ values, which are the three most important parameters associated with *A. acidoterrestris* spoilage in the present study. This means that to some extent slow-cooling conditions lead to injuries in *Alicyclobacillus* spores. We hypothesize that under slow-cooling conditions, extra energy is spent by *Alicyclobacillus* to repair the damages caused by heat processing when cooling is finished. This would be a result of longer exposure to lethal temperatures under these conditions than under quick-cooling conditions, which leads to an extension of parameters such as lag time and κ. However, as the understanding of this effect was not in the scope of the present study, it could be addressed by further studies applying molecular biology or biochemical methods.

Lag phase extension is the most important parameter on which inhibitory food preservation methods rely. Thus, an extension of this parameter consequently resulted in an increase in the time to detect guaiacol or time to orange juice spoilage. Although the two inoculum spore levels studied here were not implicated in a significant difference between λ parameters ($P < 0.05$) under the same conditions, when quick- and slow-cooling conditions are compared for the same storage temperature, i.e., 25°C or 30°C, the effect of the cooling rate can be clearly seen. For treatments at 25°C, slow cooling resulted in κ values that were identical with the values obtained with quick cooling. Fig. 2 shows that λ values for treatments 1 and 4 were extremely distinct ($P < 0.05$). Although in treatment 3 a quick-cooling condition was used (cooling to the bottle's cold point), the orange juice bottles were thereafter stored at 35°C, which is a most adequate temperature condition for the growth of an acidothermophilic bacteria such as *A. acidoterrestris*. On the other hand, in treatment 4, the orange juice bottles remained stored for 48 h at 25°C before the best condition for bacterial growth was encountered. Thus, the lower the storage temperature is in the first 48 h of cooling, the higher is the lag time of the microorganism, which, as a direct consequence, gives the longest time to initiate detectable guaiacol production ($t10^4$), which is about 130 h for treatment 6. When quick- and slow-cooling conditions at 30°C are considered, this difference is no longer evident since the lag times for treatments 2 and 1 (at 10^1 spores/ml) were not significantly different ($P > 0.05$); however, in treatment 2, the κ values were slightly higher than those obtained in treatment 1.

The fact that μ was not significantly different among treatments 1 to 4 is explained by the fact that this parameter is dependent on the current growth environment, which was 35°C of final storage for all hot-filling treatments. Differences were found only when hot-filling treatments were compared with treatment 6, in which orange juice bottles were stored at 25°C, and with treatment 4 with an inoculum level of 10^1 spores/ml.

Cooling rate affected *A. acidoterrestris* maximum population (κ) for both spore levels studied. For the inoculum level of 10^1 spores/ml in treatment 4 ($\log N/N_0 = 2.90$), κ was affected by the storage period at 25°C for 48 h (followed by storage at 35°C). This can be considered a more severe condition for *A. acidoterrestris* growth (Fig. 2) among treatments 1 to 4. Despite the final storage at 35°C, the κ value was lower than in treatment 1 ($\log N/N_0 = 3.26$), where samples were not exposed to such conditions (treatment 1).

From Fig. 3, it can be clearly seen that, despite the low efficiency of the thermal process and the surge tank maintenance simulation, the product remained stable, without guaiacol production, if the final storage was at 20°C. The literature gives conflicting results in relation to the minimum tempera-

ture for the growth of *A. acidoterrestris*. Previdi and Colla (21) found 25°C, Pettipher et al. (20) suggested 25°C, Walls and Chuyate (30) suggested 20°C, Baumgart and Menje (4) suggested 23°C, and Jensen and Whitfield (11) reported 20°C. A restricted spectrum of temperatures (between 20 and 25°C) seems to define the safe storage of orange juice. In the present work, as the levels inoculated were $<10^1$ and 10^1 spores/ml and the critical count for guaiacol production is 10^4 CFU/ml, the only remaining barrier against growth was the storage temperature at 20°C. It seems that such a storage temperature could contribute to the microbiological stability of the hot-filled orange juice during its shelf life if *A. acidoterrestris* growth is the target. This storage temperature (20°C) does not require investments as high as those required to reduce temperatures to 4°C and can be considered as a good alternative to avoid *A. acidoterrestris* growth and spoilage of orange juice.

The experimental results and predictive curves of the cold-filled conditions are shown in Fig. 4. In spite of the cold fill and incubation at 25°C, the microorganism was able to survive and germinate; this treatment provided a lag time about 2.5 times longer than treatment 6 (the worst condition for *A. acidoterrestris* growth in the context of treatments 1 to 4). Guaiacol production was not detectable by the Kirin kit since the population did not reach 10^4 CFU/ml. The mean generation time was around 16 h, which is twice that observed for the hot-fill treatments. If higher levels of inocula were used ($>10^1$ spores/ml), the maximum *A. acidoterrestris* population could be higher than 10^4 CFU/ml, leading to orange juice spoilage.

For treatment 4, varying the inoculum level showed that an inoculum of $<10^1$ spores/ml had a higher ratio ($\log N/N_0$) than 10^1 spores/ml (Fig. 5). This is a result of the same N value found in both situations. This value was not higher than 10^5 CFU/ml in any situation. Furthermore, growth rates (μ) were very similar, which shows that inoculum level does not greatly influence this parameter.

Using the Kirin kit, we observed that the time for guaiacol detection decreases with an increase in inoculum level. For instance, for treatment 2 (30°C at 48 h followed by storage at 35°C), with an inoculum of $<10^1$ spores/ml, the first positive sample for guaiacol was observed at 200 h, while with 10^1 spores/ml the first positive sample was observed at 120 h. The $t10^4$ (obtained from growth curves) values of this treatment ranged between 100 and 108 h when the inoculum level was 10^1 spores/ml and between 112 and 120 h when it was $<10^1$ spores/ml. Pettipher et al. (20) analyzed guaiacol by gas chromatography-mass spectrometry and observed that a population of 10^5 CFU/ml was also required before *A. acidoterrestris* produced enough guaiacol to cause a detectable taint in fruit juices. Although the time estimated for guaiacol production varied from 100 to 108 h when the inoculum level was 10^1 spores/ml (Table 1), the first positive result using the Kirin kit was at 120 h. This can be explained by the fact that a visual judgment is possible only above 25 ppm of guaiacol. Hence, $t10^4$ can be considered as a fail-safe value compared to Kirin kit results and an adequate parameter to avoid *A. acidoterrestris* spoilage. Although *Alicyclobacillus* sp. produces two main types of off flavors in fruit juices, guaiacol and halophenols (2,6-dibromophenol and 2,6-dichlorophenol) (6), it seems that the first is the more important metabolite produced by this microorganism. According to Siegmund and Pöllinger-Zierler (23), this compound was found in five of the eight spoiled apple juices, while 2,6-dichlorophenol was present in only one sample. However, the use in the present study of a known guaiacol-producing strain (*A. acidoterrestris* CRA 7152) (18; also J. Delves-Broughton, personal communication) shows the adequacy of the Kirin kit. It is known that the role of halophenols should not be neglected as a source of off flavors from *Alicyclobacillus* (6), but due to the unavailability of rapid kits to detect this compound when the experiments were performed, the present study focused on guaiacol detection.

In conclusion, treatment 5 (storage at 20°C) was more efficient than any of the others since in this case the *A. acidoterrestris* population remained inhibited for the entire shelf-life of the orange juice. Treatment 6 (filling and storage at 25°C) could be an alternative for spoilage control if the contamination level does not exceed 10^1 spores/ml. Since contamination control by several strategies, such as fruit disinfection and heat treatment, is not enough to ensure *A. acidoterrestris* elimination from fruit juices, hot-filled orange juice should be stored at 20°C or below when contamination with this microorganism is observed. This measure can be considered a safe, easy, and inexpensive alternative procedure to avoid *A. acidoterrestris* growth during the orange juice shelf-life.

ACKNOWLEDGMENTS

The authors acknowledge financial support provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

REFERENCES

1. **Association of the Industries of Juices and Nectars from Fruits and Vegetables of the European Union (AIJN).** 2008. *Alicyclobacillus* best practice guideline. Association of the Industries of Juices and Nectars from Fruits and Vegetables of the European Union, Brussels, Belgium. http://www.unipektin.ch/docus/public/AIJN_Alicyclobacillus_Best_Practice_Guideline_July_2008.pdf.
2. **Bahçeci, K. S., and J. Acar.** 2007. Modeling the combined effects of pH, temperature and ascorbic acid concentration on the heat resistance of *Alicyclobacillus acidoterrestris*. Int. J. Food Microbiol. **120:**266–273.
3. **Baranyi, J., and T. A. Roberts.** 1995. Mathematics of predictive food microbiology. Int. J. Food Microbiol. **26:**199–218.
4. **Baumgart, J., and S. Menje.** 2000. The impact of *Alicyclobacillus acidoterrestris* on the quality of juices and soft drinks. Fruit Process. **7:**251–254.
5. **Cerny, Y. G., W. Hennlich, and K. Poralla.** 1984. Spoilage of fruit juice by bacilli: isolation and characterization of the spoiling microorganism. Z. Lebensmitte Unters Forsch. **179:**224–227.
6. **Chang, S., and D. Kang.** 2004. *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. Crit. Rev. Microbiol. **30:**55–74.
7. **Eguchi, S. Y., G. P. Manfio, M. E. Pinhatti, E. Azuma, and S. F. Variane.** 1999. Acidothermophilic sporeforming bacteria (ATSB) in orange juices: detection methods, ecology, and involvement in the deterioration of fruit juices. ABECitrus, Campinas, Brazil. http://www.abecitrus.com.br/dload/ep_atsb.PDF.
8. **Eiroa, M. N. U., V. C. A. Junqueira, and F. L. Schmidt.** 1999. *Alicyclobacillus* in orange juice: occurrence and heat resistance of spores. J. Food Prot. **62:**883–886.
9. **Goto, K., K. Mochida, M. Asahara, M. Suzuki, H. Kasai, and A. Yokota.** 2003. *Alicyclobacillus pomorum* sp. nov., a novel thermo-acidophilic, endospore-forming bacterium that does not possess omega-alicyclic fatty acids, and emended description of the genus *Alicyclobacillus*. Int. J. Syst. Evol. Microbiol. **53:**1537–1544.
10. **International Federation of Fruit Juice Producers.** 2004. First standard IFU-method on the detection of *Alicyclobacillus* in fruit juices. IFU document IFUMB 12. International Federation of Fruit Juice Producers, Paris, France. http://www.ifu-fruitjuice.com/index_commande.php.
11. **Jensen, N., and F. B. Whitfield.** 2003. Role of *Alicyclobacillus acidoterrestris* in the development of a disinfectant taint in shelf-stable fruit juice. Lett. Appl. Microbiol. **36:**9–14.
12. **Lee, S. Y., G. I. Dancer, S. S. Chang, M. S. Rhee, and D. H. Kang.** 2006. Efficacy of chlorine dioxide gas against *Alicyclobacillus acidoterrestris* spores on apple surfaces. Int. J. Food Microbiol. **108:**364–368.

13. **Matsubara, H., K. Goto, T. Matsumura, K. Mochida, M. Iwaki, M. Niwa, and K. Yamasato.** 2002. *Alicyclobacillus acidiphilus* sp. nov., a novel thermoacidophilic, omega-alicyclic fatty acid-containing bacterium isolated from acidic beverages. Int. J. Syst. Evol. Microbiol. **52:**1681–1685.
14. **Murakami, M., H. Tedzuka, and K. Yamazaki.** 1998. Thermal resistance of *Alicyclobacillus acidoterrestris* spores in different buffers and pH. Food Microbiol. **15:**577–582.
15. **Niwa, M., and A. Kuriyama.** 2003. A. acidoterrestris: rapid detection kit. Fruit Proc. **2:**102–107.
16. **Orr, R. V., and L. R. Beuchat.** 2000. Efficacy of disinfectants in killing spores of *Alicyclobacillus acidoterrestris* and performance of media for supporting colony development by survivors. J. Food Prot. **63:**1117–1122.
17. Reference deleted.
18. **Peña, W. E. L., and P. R. Massaguer.** 2006. Microbial modeling of *Alicyclobacillus acidoterrestris* CRA7152 growth in orange juice with nisin addition. J. Food Prot. **69:**1904–1912.
19. **Petrus, R. R.** 2000. Development of a process and evaluation of isothonic beverage stability packed in aseptic plastic bottles. M.A. dissertation. State University of Campinas, Campinas, Brazil.
20. **Pettipher, G. L., M. E. Osmundson, and J. M. Murphy.** 1997. Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. Lett. Appl. Microbiol. **24:**185–189.
21. **Previdi, M. P., and F. Colla.** 1995. Characterization of *Alicyclobacillus*. A sporeforming thermophilic acidophilic bacterium. Industria Conserve **70:** 128–132.
22. **Sant'Ana, A. S., A. Rosenthal, and P. R. Massaguer.** 2009. Heat resistance and the effects of continuous pasteurization on the inactivation of *Byssochlamys fulva* ascospores in clarified apple juice. J. Appl. Microbiol. **107:**197–209.
23. **Siegmund, B., and B. Pöllinger-Zierler.** 2006. Odor thresholds of microbially induced off-flavor compounds in apple juice. J. Agric. Food Chem. **54:**5984–5989.
24. **Silva, F. V. M., and P. Gibbs.** 2004. Target selecting in designing pasteurization processes for shelf-stable high-acid fruit products. Crit. Rev. Food Sci. Nutr. **44:**353–360.
25. **Splittstoesser, D. F., C. Y. Lee, and J. J. Churey.** 1998. Control of *Alicyclobacillus* in the juice industry. Dairy Food Environ. Sanit. **18:**585–587.
26. **Stumbo, C. R.** 1965. Thermobacteriology in food processing. Academic Press, New York, NY.
27. **Tribst, A. A. L., A. S. Sant'Ana, and P. R. Massaguer.** 2009. Review: microbiological quality and safety of fruit juices: past, present and future perspectives. Crit. Rev. Microbiol. **35:**310–339.
28. **Vieira, M. C., A. A. Teixeira, F. M. Silva, N. Gaspar, and C. L. M. Silva.** 2002. *Alicyclobacillus acidoterrestris* spores as a target for Cupuacu (*Theobroma grandiflorum*) nectar thermal processing: kinetic parameters and experimental methods. Int. J. Food Microbiol. **77:**71–81.
29. **Walker, M., and C. A. Phillips.** 2007. The effects of preservatives on *Alicyclobacillus acidoterrestris* and *Propionibacterium cyclohexanicum* in fruit juice. Food Control **19:**974–981.
30. **Walls, I., and R. Chuyate.** 1998. *Alicyclobacillus*—historical perspective and preliminary characterization study. Dairy Food Environ. Sanit. **18:**499–503.
31. **Wisotzkey, J. D., P. Jurtshuk, J. R. George, E. Fox, G. Deinhard, and K. Poralla.** 1992. Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. Int. J. Syst. Evol. Microbiol. **42:**263–269.
32. **Yamazaki, K., H. Teduka, and H. Shinano.** 1996. Isolation and identification of *Alicyclobacillus acidoterrestris* from acidic beverages. Biosci. Biotechnol. Biochem. **60:**543–545.
33. **Yamazaki, K., M. Murakami, Y. Kawi, N. Inoue, and T. Matsuda.** 2000. Use of nisin for inhibition of *Alicyclobacillus acidoterrestris* in acidic drinks. Food Microbiol. **17:**315–320.